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An additive effect of elevated atmospheric CO_2 and rising temperature on methane emissions related to methanogenic community in rice paddies

Cong Wang[a,1], Yaguo Jin[a,1], Cheng Ji[a,1], Na Zhang[a], Mingyang Song[a], Delei Kong[a], Shuwei Liu[a,b], Xuhui Zhang[c], Xiaoyu Liu[c], Jianwen Zou[a,b,*], Shuqing Li[a,b,*], Genxing Pan[c]

[a] Jiangsu Key Laboratory of Low Carbon Agriculture and GHGs Mitigation,College of Resources andEnvironmental Sciences, Nanjing Agricultural University, Nanjing, China

[b] Jiangsu Key Lab and Engineering Center for Solid Organic Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing, China

[c] Center of Agriculture and Climate Change, Institute of Resource, Ecosystem and Environment of Agriculture, Nanjing Agricultural University, Nanjing, China

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ABSTRACT

Both elevated atmospheric carbon dioxide (CO_2) and rising temperature can alter soil methane (CH_4) fluxes, leading to a feedback to climate change. However, predicting this feedback needs to understand the microbial mechanisms involved in CH_4 emissions driven by climate change. A 3-year field measurement of CH_4 fluxes from rice paddies was taken in 2012–2014 to examine their responses to elevated CO_2 (enriched up to 500 μmol mol^{-1}) and rising canopy air temperature (above ambient 1.5–2.0 °C) using a free-air CO_2 enrichment (FACE) system. Using real-time PCR and Illumina MiSeq sequencing of 16S rRNA genes, we measured the abundance and composition of methanogenic community in rhizosphere soil of rice paddies in 2014. Elevated CO_2 and rising temperature showed additive effects on CH_4 fluxes and methanogen abundances, where CH_4 fluxes were correlated with methanogen abundances. Elevated CO_2, rising temperature and their combination increased seasonal CH_4 emissions by 28–120%, 38–74% and 82–143%, respectively. Either elevated CO_2 or rising temperature did not significantly alter the diversity of methanogenic community, and methanogenic genera *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, *Methanocella* and *Methanoregula* dominated in rhizosphere soils for all treatments. However, elevated CO_2 induced a shift from acetoclastic to hydrogenotrophic methanogens in their relative abundances. Rising temperature stimulated CH_4 emissions by increasing CH_4 production per individual predominant methanogen genus. Besides the enhancement of soil C substrates and rhizosphere methanogen abundances as previously reported, an additive effect of elevated CO_2 and canopy warming on CH_4 emissions is also associated with elevated CO_2-induced changes in the composition of methanogenic archaea and warming-stimulated the activity of methanogenic archaea in rice paddies.

1. Introduction

Atmospheric carbon dioxide (CO_2) and methane (CH_4) are the two major potent greenhouse gases (GHGs). Increasing concentrations of GHGs for 2011 relative to 1750 have contributed to an anthropogenic radiative forcing of 2.83 W m^{-2}, where CO_2 and CH_4 account for 1.68 W m^{-2} and 0.97 W m^{-2}, respectively (IPCC, 2013). As a consequence of continuing buildup of GHGs in the atmosphere, increases in global surface air temperature between the mid-21 st century and the reference period of 1986–2005 are projected to likely exceed 1.4 °C and 2.0 °C for RCP4.5 and RCP 8.5, respectively (IPCC, 2013). Both elevated atmospheric CO_2 and rising temperature can alter soil GHG fluxes, leading to a feedback to climate change (Frank et al., 2010; van Groenigen et al., 2011; Gaihre et al., 2014). Predicting this feedback needs to understand the mechanisms and processes involved in GHGs emission under climate change scenarios (Schimel and Gulledge, 1998; Conrad, 2007). However, the combined effects of elevated CO_2 and rising temperature on soil carbon and nitrogen biogeochemistry and soil GHGs emission remain poorly understood (Dijkstra et al., 2012; Liu et al., 2012; Yue et al., 2017).

Rice paddies play an important role in atmospheric GHGs budget, accounting for 10% of total anthropogenic CH_4 emissions or about 1.5%

* Corresponding authors at: Jiangsu Key Laboratory of Low Carbon Agriculture and GHGs Mitigation, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, China

E-mail addresses: jwzou21@njau.edu.cn (J. Zou), shuqingli@njau.edu.cn (S. Li).

[1] Contributed equally to this paper.

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of global gross GHGs emissions (FAOSTAT, 2014). While rice production responses to climate change are of great concern (Ainsworth and Long, 2005; Wang et al., 2015, 2016; Cai et al., 2016), recent attention has been increasingly directed towards the effects of climate change on CH_4 emissions from rice paddies to understand its feedback to climate change. By summarizing available field measurements, a meta-analysis synthesized that elevated atmospheric CO_2 incurred, on average, an increase of 43% in CH_4 emissions from rice paddies (van Groenigen et al., 2011). However, the magnitude of this effect varies widely with many other factors, such as soil property, water management regime, crop residue amendment, fertilizer application, rice cultivar and plant growth, and experimental method (Allen et al., 2003; Inubushi et al., 2003; Xu et al., 2004; Zheng et al., 2006; van Groenigen et al., 2011).

Numerous experiments have been carried out to examine the response of CH_4 emissions to elevated atmospheric CO_2 in controlled-environment chambers and in the field FACE (Free-Air-CO_2-Enrichment) systems (Ziska et al., 1998; Allen et al., 2003; Inubushi et al., 2003; Xu et al., 2004; Zheng et al., 2006; Tokida et al., 2010; Liu et al., 2012). The effects of increased soil temperature on CH_4 emissions from paddy soils are examined mostly in laboratory incubation experiments, while very few studies have concentrated on the effects of canopy air temperature on CH_4 emissions in the field (Allen et al., 2003; Gaihre et al., 2014). It is generally believed that CH_4 fluxes response to elevated CO_2 is more realistic under field FACE experiments than under phytotron or greenhouse conditions. Rice growth and yield responses to elevated CO_2 are less in field FACE experiments than in the controlled enclosure experiments (Ainsworth and Long, 2005; Wang et al., 2015; Cai et al., 2016), which would incur a difference in CH_4 fluxes response to elevated CO_2 between the two experiment methods.

To examine the combined effect of elevated CO_2 and rising temperature on CH_4 emissions, almost all FACE field experiments were designed with increased soil temperature (Tokida et al., 2010, 2011; Liu et al., 2012), while elevated CO_2 combined with rising air temperature was designed only in two studies, one in controlled-environment chambers (Allen et al., 2003) and the other one in open-top chambers (OTCs) (Ziska et al., 1998). The response of CH_4 emissions to rising soil temperature may differ with their response to rising canopy air temperature in rice paddies (Ziska et al., 1998; Allen et al., 2003; Liu et al., 2012; Gaihre et al., 2014). In rice paddies, flood water and soil temperature can track the controlled air temperature closely during the initial flooding and midseason drainage stages when there is little shading of the water surface by rice vegetation (Allen et al., 2003). During the late season with complete rice vegetative cover, soil temperature can hardly increase with canopy warming (Ziska et al., 1998; Gaihre et al., 2014; Liu et al., 2016a). Since elevated atmospheric CO_2 is accompanied by an increase in air temperature under future climate change, nevertheless, the experiments of elevated CO_2 combined with rising canopy air temperature deserve to be highlighted, particularly under field conditions.

The interactive effects of multiple global change factors on soil C and N biogeochemistry may be additive (i.e., not differing from the sum of their individual effects), non-additive synergistic or non-additive antagonistic (Dijkstra et al., 2012; Yue et al., 2017). By synthesizing available data, a meta-analysis concluded that the interactive effects of elevated CO_2 and rising temperature on soil C pools are generally additive (Yue et al., 2017). However, the interactive effects of elevated CO_2 and rising temperature on CH_4 emissions from rice paddies have been rarely examined and very few available studies have generated contradictory results (Dijkstra et al., 2012). A non-additive synergistic effect of elevated CO_2 and rising temperature on CH_4 emissions was found in controlled-environment chamber studies (Allen et al., 2003) or laboratory soil microcosms (Das and Adhya, 2012), while an additive effect of elevated CO_2 and rising soil/air temperature on CH_4 emissions was reported in field OTC/FACE studies (Ziska et al., 1998; Liu et al., 2012). Nevertheless, more field FACE studies under simulating atmospheric CO_2 enrichment combined with rising air temperature are highly needed to reconcile this debate.

Given that CH_4 is produced by methanogenic archaea in rice paddies, it is important to understand how methanogenic archaea abundance and community structure influences CH_4 production under climate change scenarios (Schimel and Gulledge, 1998; Conrad, 2007). Some studies showed that elevated atmospheric CO_2 and rising temperature can alter the soil, rhizosphere or root microbial community (Yue et al., 2007; Peng et al., 2008; Das and Adhya, 2012; Lu et al., 2015; Liu et al., 2016a), while their effects on the abundance and composition of methanogenic community were not significant in field FACE studies (Angel et al., 2012; Liu et al., 2012, 2016b). Nevertheless, insights into responses of methanogenic community to elevated CO_2 and rising temperature in rice paddies are remained poor (Alpana et al., 2017). In particular, rising canopy air temperature effects and the interactive effects with elevated CO_2 on methanogenic community have not been linked to CH_4 fluxes (Singh et al., 2010; Liu et al., 2016a; Alpana et al., 2017). Our FACE experiment under atmospheric CO_2 enrichment combined with rising air temperature would help to examine their combined effects on the methanogenic abundance and structure and the consequences for CH_4 production in rice paddies.

Here, we first presented field measurements of CH_4 flux from rice paddies under elevated CO_2 and rising canopy temperature in a FACE system over the 2012–2014 seasons. The FACE system under elevated atmospheric CO_2 (up to 550 μmol mol^{-1}) combined with rising canopy temperature (1.5–2.0 °C above ambient) has been established in rice paddies in southeast China since 2011. To understand methanogenic community linking to seasonal CH_4 flux responses to elevated CO_2 and rising temperature, the abundance and composition of methanogenic community were measured in rhizosphere soil of rice paddies over the 2014 rice-growing season. The main objective of this study is to examine the combined effect of elevated CO_2 and rising canopy temperature on CH_4 emissions related to methanogenic community in rice paddies. Specifically, we attempted to address the following concerns: (i) Whether the interactive effects of elevated CO_2 and rising air temperature on CH_4 emissions are additive, non-additive synergistic or non-additive antagonistic in rice paddies? (ii) How do combined elevated CO_2 and rising air temperature affect methanogenic community (abundance, composition and activity) in rice paddies? (iii) Whether CH_4 fluxes are related to methanogenic community over the rice-growing season under FACE systems?

2. Materials and methods

2.1. Experiment site

A field FACE experiment over the 2012–2014 rice seasons was carried out in rice paddies on the experimental farm of Nanjing Agricultural University, Changshu, Jiangsu province, China (31°30′N, 120°33′E). The field site is located in the center of the Tai Lake Plain region, where cropping regime is overwhelmingly dominated by an annual paddy rice-winter wheat rotation system. The region displays a typical monsoonal climate, where seasonal mean temperature was 22.0 °C in 2012, 27.0 °C in 2013 and 24.2 °C in 2014. Seasonal rainfall totaled 1045 mm in 2012, 680 mm in 2013 and 760 mm in 2014. Soil of the experimental site is classified as Gleyic Stagnic Anthrosol, which is developed from clayey lacustrine deposit and under paddy rice-wheat rotation cultivation for hundreds of years. Prior to the 2012 rice season, topsoil pH, bulk density, organic C and total N contents were 6.7 (1:2.5, water/soil, w/w), 1.20 g cm^{-3}, 15.0 g kg^{-1} and 1.6 g kg^{-1}, respectively.

2.2. Rice cultivation

All the experimental plots were in line with the local typical cultivation practices including rice cultivar, water and fertilization regimes in rice paddies in 2012–2014. Rice (*Oryza sativa* L. cv. Changyou 5)

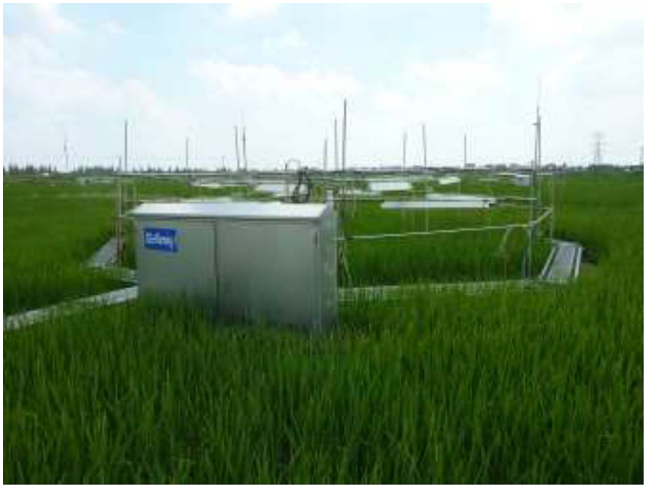

Fig. 1. A picture of field experimental plots set-up in rice paddies under FACE systems. The FACE system included twelve octagonal rings with each ring area of 50 m^2. In the CO_2 exposure system, CO_2 pumping was cycled around the octagon and the CO_2 gas was injected into the ring plot (50 m^2) via perforated pipes from a liquid CO_2 tank. In the heating system, the infrared heating facilities consisting of 12 ceramic infrared heaters (IR) supplied 140 W m^{-2} of infrared radiation to warm the canopies within the inner circle (25 m^2) of the heated plots.

seeds were sown on a nursery bed on 25–28 May and grown under ambient air until seedling transplanting at a three-leaf-stage. Rice seedlings were manually transplanted into paddy fields on 15–23 June and harvested on 20–28 October (Cai et al., 2016; Wang et al., 2016). Rice transplanting density was three seedlings per hill, and spacing of hills was 15.3 × 25.4 cm, being equivalent to 25.7 hills m^{-2} or 77.1 plants m^{-2}. All the experimental plots were protected by border plants with the similar density. A local water regime (F-D-F-M) of flooding-midseason drainage-reflooding-intermittent moist irrigation was adopted for all the field plots. Specifically, flooding was started 3–5 days before transplanting, and continued for 30–35 days until an episode of the midseason drainage for 7–10 days. Thereafter, paddy field was re-flooded until 80–90 days after transplanting and followed by intermittent moist irrigation but without waterlogged for rice harvesting. The flooding water depth was kept at 5–8 cm during the period of waterlogging in rice paddies. Urea as N fertilizer was identically broadcasted at the rate of 181 kg N ha^{-1} on all the field plots, with a split of 40% of the total as basal fertilizer before seedling transplanting, 30% on 30 days after transplanting and 30% on 45–55 days after transplanting in each rice season. Calcium superphosphate (60 kg P_2O_5 ha^{-1}) and potassium chloride (120 kg K_2O ha^{-1}) as the basal fertilizers were identically applied for all the experimental plots in each season. In addition, pesticide and herbicide management in rice season followed the local practices.

2.3. Experimental treatments and FACE system

The free-air CO_2 enrichment and temperature elevation system (FACE) has established since April 2011 (Fig. 1). The FACE system included twelve octagonal rings with each ring area of 50 m^2 established in different blocks in a rice paddy field. In the inner circle area of 25 m^2 per plot, there was a full two factorial design in the FACE system, consisting of four treatments with three replicates. The four experimental treatments included atmospheric CO_2 concentration target for 500 μmol mol^{-1} (C), warming of canopy air temperature by 1.5–2.0 °C above ambient (T), the combined CO_2 enrichment and warming (C + T), and the control under ambient conditions (Ambient). Three replicates of each treatment were established in three ring plots with the identical infrastructure. To avoid the cross-over effect of treatments, each treatment ring plot was isolated by an adjacent field block. Over the 2012–2014 rice-growing seasons, the ambient CO_2 concentration above the canopy was 392–430 μmol mol^{-1} and canopy air temperature was 21.5–23.4 °C. The CO_2 concentration and canopy temperature of the experimental treatments relative to the ambient control were detailed in Table 1.

The design of CO_2 exposure system and the infrared heating facilities was complied with the rationales proposed by Okada et al. (2001) and Kimball et al. (2008), respectively (Fig. 1). Schematic diagram of the FACE system was shown in Cai et al. (2016) and Wang et al. (2016). In the CO_2 exposure system, CO_2 pumping was cycled around the octagon and the CO_2 gas was injected into the ring plot (50 m^2) via perforated pipes from a liquid CO_2 tank (purity 99.99%). The CO_2 pumping in each plot was automatically controlled by sixteen Li-820 CO_2 sensors (Li-COR Inc., Lincoln, NE, USA) installed above the canopy and evenly distributed in two concentric circles. In the heating system, the infrared heating facilities consisting of 12 ceramic infrared heaters (IR) (2000 W, 240 V, 1.65 m long × 0.14 m wide; HS-2420, Kalglo Electronics Co., Inc., Bethlehem, PA, USA) supplied 140 W m^{-2} of infrared radiation to warm the canopies within the inner circle (25 m^2) of the heated plots. We manually adjusted the heaters once a week so as to keep their height being 1.2 m above the canopy over the whole rice season. The CO_2 exposure system and heating system worked both daytime and nighttime. A datalogger (CR 1000; Campbell Scientific Inc.) automatically monitored and recorded canopy temperature and atmospheric CO_2 concentration above the canopy every 1 min. Details of the FACE system design, facilities, operation and performance were available in previous and related publications (Liu et al., 2014; Yang et al., 2015; Cai et al., 2016).

2.4. CH_4 flux measurements

The CH_4 fluxes were in situ measured using the static chamber-gas chromatograph (GC) method (Zou et al., 2005; 2009). Prior to the initial field flooding, three pottery-made cylindrical flux collars (0.30 m in diameter × 0.20 m height) were permanently installed for each plot to ensure reproducible gas flux measurements over the whole experimental period. The open-bottomed PVC chamber was 1.0 m high and had the same cross-sectional area as collar base. While gas sampling, the chamber was placed over the rice vegetation, and the rim of the chamber was sealed by water filling into a groove (5 cm in depth) at the top edge of the collar base. Gas flux measurements were taken once a week except for measurements once a day during the mid-season drainage period. Gas samples were collected from inside the chambers using 60-mL plastic syringes fitted with three-way stopcocks. Gas samples were taken between 0800 and 1000 LST on each sampling day (Zou et al., 2005, 2009; Shang et al., 2011), and they were analyzed by

Table 1
Summary of daily CO_2 concentration and canopy temperature increment (Mean ± SD) of FACE treatments relative to ambient in the 2012–2014 rice-growing seasons.

Treatment	CO_2 concentration (μmol mol^{-1})			Canopy temperature (°C)		
	2012	2013	2014	2012	2013	2014
C	101 ± 22	113 ± 30	109 ± 35	–	–	–
T	–	–	–	2.0 ± 0.4	1.3 ± 0.7	2.0 ± 1.0
C + T	93 ± 20	106 ± 22	82 ± 29	1.9 ± 0.8	1.4 ± 0.9	1.9 ± 0.9

GC within a few hours.

The mixing ratio of CH_4 was analyzed using a gas chromatograph (Agilent 7890) equipped with a flame ionization detector (FID). The oven was operated at 55 °C, and the FID at 200 °C. The carrier gas (N_2) flow rate was 30 mL min^{-1}. The configuration of GC and procedure for measuring CH_4 fluxes were detailed in our previous studies (Zou et al., 2005, 2009; Qin et al., 2010). Fluxes were calculated by the slope of the mixing ratio change in five gas samples, collected at 0, 5, 10, 15 and 20 min following chamber closure. Mean of fluxes taken from the triplicate collars within each ring represent flux measurements of the field plot. Average fluxes and standard deviations of CH_4 for each treatment were calculated from triplicate rings. Seasonal total of CH_4 emissions was sequentially accumulated from the flux measurements between every two adjacent temporal intervals (Zou et al., 2005; Qin et al., 2010; Shang et al., 2011).

2.5. DNA extraction and real-time PCR assay

In the 2014 rice growing season, we collected rhizosphere soil samples during the initial flooding (July 6), onset of midseason drainage (July 21), and re-flooding (August 30) stages in each experimental plot. The rhizosphere soil samples (being tightly adhered to the plant roots with about 1 cm thickness) were randomly collected from five individual rice plants at 0–15 cm depth in each plot. The rhizosphere soil was evenly mixed to form a composite sample. Fresh samples stored at 4 °C were used to analyze soil physicochemical properties within one week of sampling (Table S1). A sub-sample of rhizosphere soil (1.0 g) derived from the middle of the composite sample was used for DNA extraction. Total genomic DNA samples were extracted by using PowerSoil DNA Isolation Kits according to the manufacturer's instruction (MoBio, CA, USA). For real-time PCR assay and MiSeq analysis, the concentration and quantity (ratio of A260/A280) of DNA samples were determined with agarose gel and a spectrophotometer (NanaDrop ND2000, ThermoScientific, DE, USA).

Real-time quantification of 16S rRNA genes of the methanogen was carried out in a StepOne™ real-time PCR system (Applied Biosystems, Germany). The q-PCR amplifications were performed in a total volume of 20 μL using a SYBR@ Premix Ex Taq™ (Takara, China), with reaction mixture containing 10 μL SYBR@ Premix Ex Taq™, 0.4 μL each primer (10 μmol L^{-1}), 0.4 μL ROX reference dye II (50 ×), 2 μL template DNA and 6.8 μL sterile water. The amplified fragments for each gene were cloned in pMD 18-T vector and sequenced. The standard curves of all genes were prepared by using triplicate 10-fold dilutions of linear plasmid DNA. Amplification was performed triplicates under the following cycling conditions: 30 s at 95 °C, sequenced by 40 cycles of 95 °C, 60 °C and 72 °C for 5 s, 34 s, and 15 s, respectively, and eventually a dissociation stage (95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s).

2.6. Illumina MiSeq sequencing of 16S rRNA genes

The PCR amplification of 16S rRNA genes of the methanogen from the genomic DNA was performed using a universal primer set 1106F/1378R (Feng et al., 2013). Triplicate PCR products of each DNA sample were combined and separated by 2% agarose gel electrophoresis. The targeted bond (approximately 273 bp) was purified using the Axyprep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Madison, WI, USA). The equimolar purified amplicons were pooled and pair-end sequenced (2 × 250) on the Illumina MiSeq platform according to the standard protocols at Shanghai BIOZERON Biotechnology Co., Ltd. (Shanghai, China). The Illumina MiSeq sequencing data are available in the NCBI Sequence Read Archive (SRA) database under accession number SRP093483.

2.7. Illumina data analysis

After sequencing was completed, three criteria were followed for demultiplexing and quantity-filtering the raw fastq files by Quantitative Insights Into Microbial Ecology (QIIME) (Version 1.17) (Caporaso et al., 2010). Sequences with a quantity score below 25 and a length fewer than 200 bp were eliminated. Sequences were binned into operational taxonomy units (OTUs) using a 97% identity threshold. Chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). The phylogenetic affiliation analysis of each 16S rRNA gene sequence was introduced by RDP Classifier against the silva 104 database (http://www.arb-silva.de/download/archive/qiime/) with confidence threshold of 70%.

The abundance-based coverage estimator (ACE), Chao1 estimator, Shannon diversity and Good's coverage were calculated to examine the alpha-diversity of methanogen community. For beta-diversity, Mothur (Schloss et al., 2009) based on the OTU composition was performed to draw hierarchical cluster dendrograms (with Bray-Curtis distance dissimilarities) so as to compare the community structures across all samples. The QIIME was used to calculate the weighted pairwise UniFrac distances (Lozupone and Knight, 2005) for community comparisons, which were visualized using non-metric multidimensional scaling plots in PRIMER v6 (Clarke and Warwick, 2001). To test the significance of factors accounting for the divergence in methanogenic archaea communities, a permutational multivariate analysis of variance (PERMNOVA) was conducted using the Adonis function in R vegan (Oksanen et al., 2007) package with 999 permutations. Redundancy analysis (RDA) was carried out using CANOCO for Windows to visualize the relationship between methanogenic archaea communities and environmental factors.

2.8. Statistical analysis

A three-way analysis of variance was performed to test the differences in seasonal CH_4 emissions over the 2012–2014 period as affected by CO_2 fumigation, warming, year and their interactions (ANOVA, Table 2). The effects CO_2 fumigation, warming, stage and their interactions on methanogen abundance and relative abundance of methanogen genus were examined by using a repeated measures analysis of variance (MANOVA, Table 3). Statistical procedures were performed with the SPSS 22.0 software package for windows and *R* (Team, 2012). Regression of CH_4 fluxes with methanogen abundances and the relative abundances of *Methanosaeta* genus was fitted by a linear model with the personality of ordinary least squares (OLS).

3. Results

3.1. CH_4 flux

Seasonal pattern of CH_4 fluxes did not differ among the experimental years and the field treatments, while it depended on water regime (Fig. 2). During the period of initial flooding, CH_4 emissions increased steadily to the largest fluxes on approximately 25–30 days after seedling transplanting. Thereafter, one-week midseason drainage incurred a dramatic decrease in CH_4 fluxes and then remained at a lower emission rate. Overall, there were similar temporal trends but differing in amplitudes of CH_4 fluxes among the treatments during the 2012–2014 rice-growing seasons (Fig. 2).

Seasonal total of CH_4 emissions depended greatly on elevated CO_2 and rising temperature and varied with year, while it was not significantly affected by their interactions (Table 2). Under an identical experimental treatment, seasonal total of CH_4 emissions in 2013 was significantly greater than that in the 2012 and 2014 seasons (Table 2). On average, seasonal CH_4 emissions ranged from 51.39 kg CH_4 ha^{-1} for the ambient control plots in 2012 to 138.17 kg CH_4 ha^{-1} for the C + T plots in 2013. For the control plot under ambient conditions, seasonal

Table 2
Seasonal CH_4 emissions (kg ha^{-1}) from rice paddies under elevated atmospheric CO_2 (C) and rising temperature (T) in 2012–2014 and results of a three-way analysis of variance (ANOVA).

Treatments	2012	2013	2014
Ambient	51.39 ± 5.34	75.78 ± 9.08	60.94 ± 7.82
Elevated CO_2 (C)	113.16 ± 23.65	121.75 ± 12.28	77.77 ± 10.32
Rising temperature (T)	89.26 ± 9.18	104.68 ± 13.82	89.26 ± 8.14
C + T	125.08 ± 19.92	138.17 ± 14.87	110.81 ± 17.64

ANOVA

Effect	*SS*	*F-value*	*P-value*
C	6.45	51.57	< **0.0001**
T	3.40	27.20	< **0.001**
Year (Y)	4.24	14.85	**0.003**
C × T	0.16	1.26	0.29
C × Y	1.01	3.54	0.09
T × Y	0.10	0.36	0.71
C × T × Y	0.24	0.82	0.47
Model	10.01	26.68	< **0.001**

The significance at statistical level of 0.05 for bold values.

CH_4 fluxes averaged 42.8 mg m^{-2} day^{-1} in 2012, 62.6 mg m^{-2} day^{-1} in 2013, and 49.1 mg m^{-2} day^{-1} in 2014. Relative to the ambient control, elevated CO_2 significantly increased seasonal CH_4 emissions by 120%, 28% and 61% in the 2012, 2013 and 2014 seasons, respectively (Table 2). In comparison with the ambient control, rising canopy temperature increased seasonal CH_4 emissions by 38–74% in 2012–2014, with an average flux of 72.0–86.5 mg m^{-2} day^{-1}. When elevated CO_2 and rising canopy temperature were simultaneously simulated, seasonal CH_4 fluxes averaged 89.4–114.2 mg m^{-2} day^{-1} for the C + T plots in 2012–2014, 82–143% greater than the control under ambient conditions (Table 2).

3.2. Methanogen abundance

The abundance of methanogen showed a pronounced variation over the rice-growing season (Fig. 3). Specifically, methanogen abundances were lower when the field was initially flooded, and they were the greatest at the onset of midseason drainage. Methanogen abundances were decreased following one-weak midseason drainage episode and thereafter remained at relatively lower levels when the field was re-flooded. Over the 2014 rice-growing season, both elevated CO_2 and rising temperature had significant effects on methanogen abundances, but their interaction was not pronounced (MANOVA, Fig. 3). There was a significant interaction of temperature with stage on methanogen abundances (Fig. 3).

Relative to the control under ambient conditions, elevated CO_2 and rising temperature increased methanogen abundances by 17% and 9% at the initial flooding stage, 77% and 137% at the onset of midseason drainage, and 49% and 74% at the reflooding stage, respectively. Compared with the ambient control, elevated CO_2 combined with rising temperature resulted in an increase of 31–156% in methanogen abundances for the C + T plots (Fig. 3). A significant correlation of CH_4 fluxes to methanogen abundances reveals that CH_4 fluxes depended greatly on methanogen abundances in rice paddies under FACE (Fig. 4a). Besides methanogen abundance, the relative abundance of *Methanosaeta*, the most predominant methanogen genus, was found to be significantly correlated to CH_4 fluxes, revealing that the increase in the proportion of predominant methanogen genus contributed to the increase in CH_4 fluxes from rice paddies (Fig. 4b).

3.3. Methanogenic community composition

The top-20 abundant methanogenic genus OTUs in rhizosphere soils as revealed by MiSeq high-throughput sequencing belonged to methanogenic genus of *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, *Methanocella* and *Methanoregula* (Tables 3 and 4, Fig. 5). These 20 OTUs together accounted for approximately 80% of the methanogenic community, of which *Methanosaeta* (25–42%), *Methanosarcina* (13–25%), and *Methanobacterium* (9–18%) were identified to be the three most predominant taxes (Fig. 5). The relative abundance of dominant methanogen genus varied over rice-growing stages, while there were no any significant interactive effects of CO_2, temperature and stage (Table 3). Instead, elevated CO_2 significantly affected the composition of methanogenic community by decreasing the relative abundance of *Methanosaeta* and *Methanoregula* while increasing relative abundance of *Methanobacterium* and *Methanocella* (Table 3, Fig. 5). Rising canopy

Table 3
Statistics (*F*-/*P*-values) of repeated measures analysis of variance (MANOVA) for the diversity of methanogenic community and the relative abundance of predominant methanogen genus under different water management stages (S) as affected by elevated CO_2 (C) and rising temperature (T) in a rice FACE system.

Methanogen	C	T	C × T	S	C × S	T × S	C × T × S
Community diversity							
ACE	1.54/0.23	1.46/0.24	0.52/0.48	15.76/ < **0.001**	2.18/0.14	0.00/1.00	0.38/0.69
Chao1	11.03/**0.003**	0.75/0.40	0.03/0.86	18.76/ < **0.001**	5.15/**0.01**	3.42/**0.049**	0.34/0.71
Shannon	0.03/0.86	1.00/0.33	0.31/0.58	52.02/ < **0.001**	1.61/0.22	0.05/0.96	0.26/0.78
Genus relative abundance							
Methanosaeta	7.40/**0.02**	0.01/0.92	2.05/0.17	26.15/ < **0.001**	0.74/0.49	0.53/0.60	0.11/0.90
Methanosarcina	0.04/0.85	0.36/0.56	1.36/0.25	5.12/**0.01**	1.88/0.17	0.17/0.85	0.06/0.94
Methanobacterium	22.55/ < **0.001**	6.87/**0.02**	3.41/0.08	5.71/**0.009**	1.69/0.21	0.30/0.75	1.69/0.21
Methanocella	48.25/ < **0.001**	2.89/0.10	0.26/0.61	10.81/ < **0.001**	0.06/0.94	0.27/0.77	2.02/0.15
Methanoregula	3.97/**0.05**	0.68/0.42	0.10/0.75	38.93/ < **0.001**	0.68/0.52	0.30/0.74	0.03/0.96

The significance at statistical level of 0.05 for bold values.

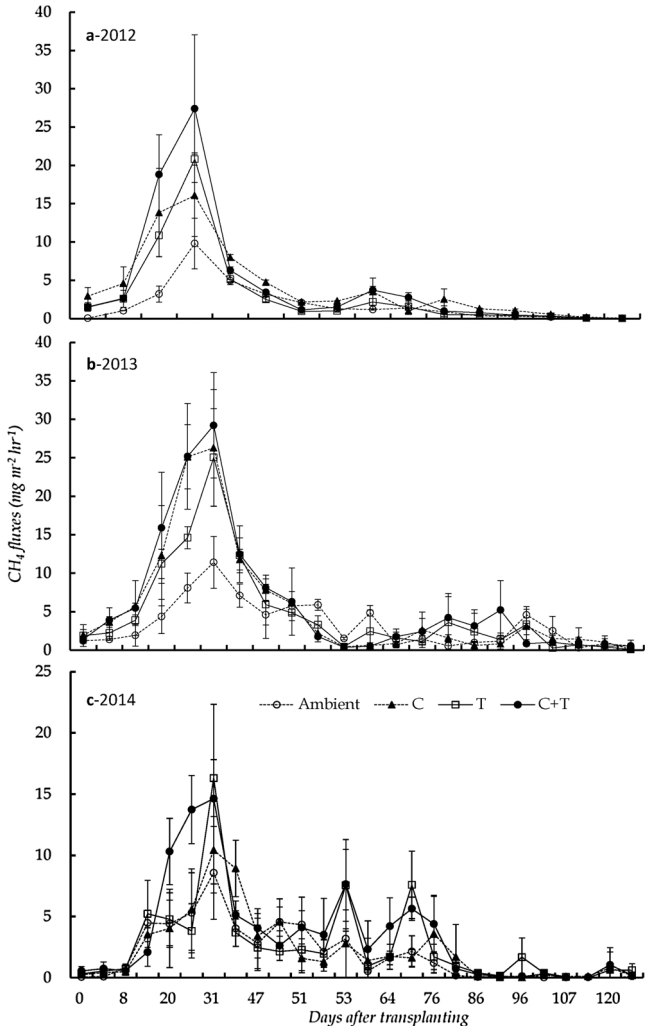

Fig. 2. Seasonal dynamics of methane fluxes from rice paddies as affected by elevated atmospheric CO_2 (C) and rising canopy temperature (T) under FACE systems in 2012–2014.

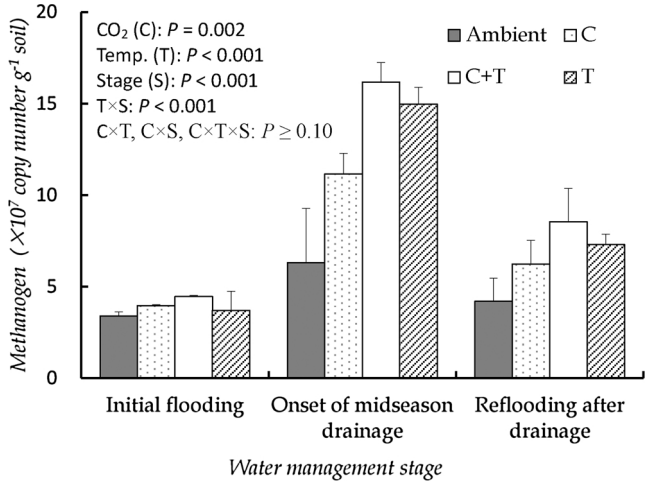

Fig. 3. Rhizosphere methanogen abundance as affected by elevated atmospheric CO_2 (C) and rising canopy temperature (T) during different water management stages over the 2014 rice-growing season.

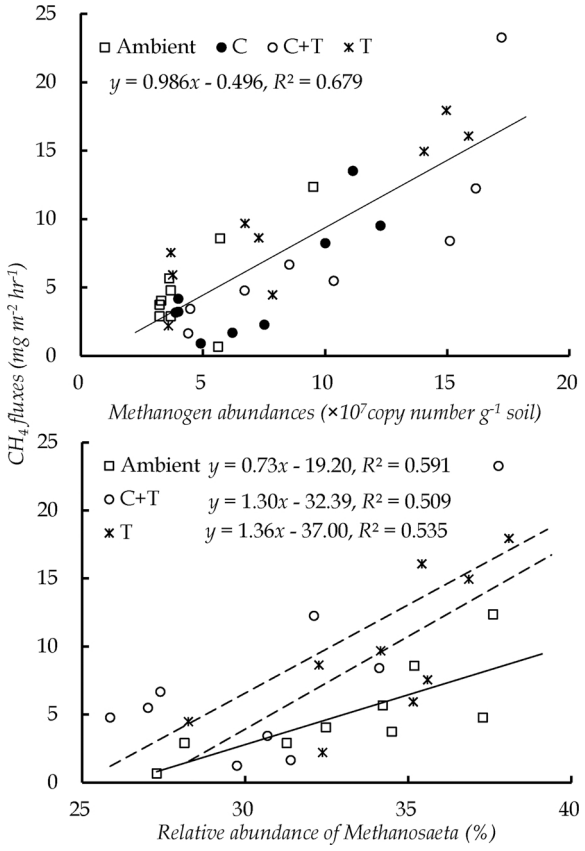

Fig. 4. Dependence of CH_4 fluxes on methanogen abundance (a) and relative abundance of *Methanosaeta* (b) in rhizosphere of rice paddies under elevated atmospheric CO_2 (C) and rising canopy temperature (T).

temperature did not significantly alter the relative abundance of methanogen genus, except for the genus *Methanobacterium* (Table 3). Instead, the regression of CH_4 fluxes with the relative abundance of methanogen *Methanosaeta* had a significantly steeper slope (1.30/1.36) in the warming plot (T and C + T) than in the control plot (0.73) (ANCOVA, interaction term, $p < 0.01$, Fig. 4b). The steeper slope of the regression line implies that warming-simulated CH_4 emissions could also due to an increase in CH_4 production efficiency of predominate methanogen.

Either elevated CO_2 or rising temperature did not significantly alter the overall distribution pattern of methanogenic community under genus level (Fig. 5). The α-diversity of methanogens in paddy soils, including ACE and Shannon, was not significantly affected by elevated CO_2, rising temperature and their interaction, but it varied with rice-growing stage (Table 3). Significant effects of CO_2 and the interactive effects of CO_2 and temperature with stage were found only for Chao1, but not for ACE or Shannon-indexes (Table 3).

The permutational multivariate analysis of variance suggested that both the experimental climate factors ($F = 6.87$, $p < 0.001$) and stage ($F = 11.36$, $p < 0.001$), but not their interaction, were responsible for the variation of the methanogenic archaeal communities in terms of the relative abundance of OTUs. The RDA biplot revealed that 37.5% of the total variability was explained by the first two axes of RDA (Fig. S1). The methanogenic communities of ambient and rising temperature treatment (T) were grouped separately from those of elevated CO_2 treatments (C and C + T) (Fig. S1). The pairwise correlation showed that soil organic matter (OM) contributed most to the difference in methanogenic community structure among the treatments, and CH_4

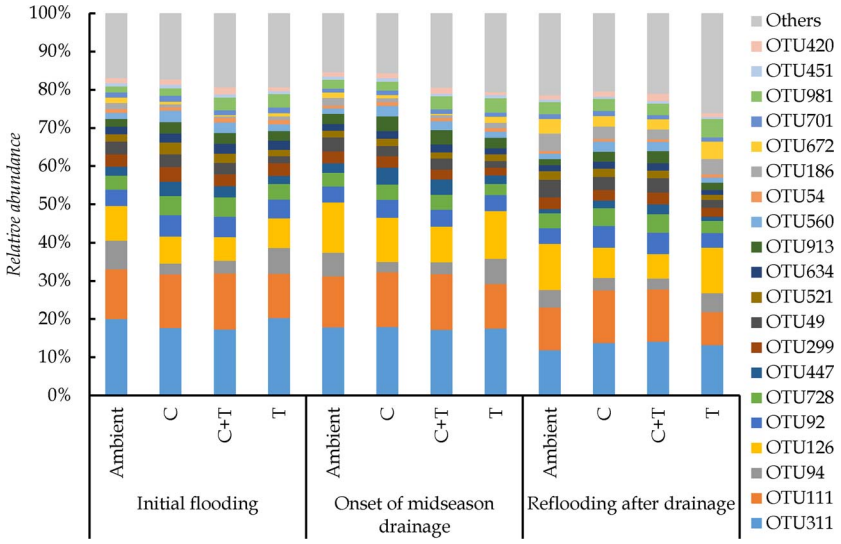

Fig. 5. Relative abundance of rhizosphere methanogen genus OTUs during different water management stages in rice paddies under elevated atmospheric CO_2 (C) and rising canopy temperature (T).

fluxes and soil NO_3^- also contributed to the difference in methanogenic community composition, while the contribution of soil NH_4^+ and C/N ratio was not pronounced (Fig. S1). Compared with the ambient control, indeed, both elevated CO_2 and rising temperature significantly enhanced soil organic matter content over the rice-growing seasons (Table S1). Together, these results suggested that elevated CO_2 and rising temperature enhanced soil C input, leading to changes in methanogenic community abundance and structure that contributed to CH_4 fluxes.

4. Discussion

4.1. An additive effect of elevated CO_2 and rising temperature on CH_4 fluxes

In this FACE field studies over the 2012–2014 rice growing seasons, seasonal pattern of CH_4 fluxes did not vary with year and experimental treatments, as reported in previous rice FACE studies in China and Japan (Inubushi et al., 2003; Xu et al., 2004; Zheng et al., 2006; Tokida et al., 2010). It is generally believed that seasonal pattern of CH_4 fluxes depends on water regime in rice paddies (Zou et al., 2005, 2009). Seasonal CH_4 fluxes from the ambient control totaled 51.39–75.78 kg CH_4 ha^{-1}, with an average flux of 42.8–62.6 mg m^{-2} day^{-1} in 2012–2014, falling well within the range of our earlier measurements in rice paddies in this area (Zou et al., 2005, 2009; Liu et al., 2010). On average, greater seasonal CH_4 emissions in 2013 relative to the 2012 and 2014 seasons would be ascribed to warmer season in 2013 with higher seasonal mean air temperature (27.0 vs. 22.0–24.2 °C) and less seasonal rainfall (particularly during midseason drainage period) and greater rice growth in the 2013 season (Cai et al., 2016).

Elevated CO_2 and rising temperature significantly increased seasonal CH_4 emissions over the 2012–2014 seasons, while their interactions were not pronounced, representing an additive effect of elevated CO_2 and rising canopy temperature on CH_4 emissions from rice paddies (Ziska et al., 1998; Tokida et al., 2010). Compared with the control under ambient conditions, elevated CO_2 significantly increased seasonal CH_4 emissions by 28–120% over the 2012–2014 rice growing seasons (Table 2). The increment of seasonal CH_4 emissions under elevated CO_2 was generally comparable to results of rice FACE studies under a similar water regime in China (30–200%, Xu et al., 2004; 38–188%, Zheng et al., 2006) and in Japan (38–51%, Inubushi et al., 2003). By summarizing available data, a meta-analysis estimated that elevated atmospheric CO_2 increased CH_4 emissions from rice paddies by 43% on average, with large variation among individual FACE studies (van Groenigen et al., 2011).

The enhancement of CH_4 emissions under elevated CO_2 is generally believed to be closely associated with the elevated CO_2-induced increase in crop growth and soil organic C input in rice paddies (Table S1; Ziska et al., 1998; Tokida et al., 2010; Liu et al., 2012). Indeed, elevated CO_2 significantly grain yield of rice by 5–8% over the 2012–2014 seasons (Cai et al., 2016). Since soil organic C buildup is also affected by agricultural practices, such as water regime, crop residue management, and N fertilizer application, the effect of elevated CO_2 on CH_4 emissions would be regulated by these agricultural practices in rice paddies. For example, some previous FACE studies showed that continuous waterlogging could weaken the increase in root mass under elevated CO_2, leading to an insignificant increase in CH_4 emissions under elevated CO_2 (Tokida et al., 2010; Liu et al., 2012), contrary to the results of rice FACE studies with midseason drainage (Table 2; Inubushi et al., 2003; Xu et al., 2004; Zheng et al., 2006). In addition, the positive effect of elevated CO_2 on CH_4 emissions would be enlarged in rice paddies where crop residues were retained in fields or N fertilizer was applied at high rates (Zheng et al., 2006).

A great many studies have used soil warming experiments to examine rising temperature effects on CH_4 emissions from rice paddies, and greater CH_4 emissions were reported in most studies (Ziska et al., 1998; Tokida et al., 2010; Liu et al., 2012). However, this effect has rarely been addressed by simulating canopy air warming in FACE studies. In this study, rising canopy temperature above the ambient 1.5–2.0 °C increased seasonal CH_4 emissions by 38–74% over the 2012–2014 rice growing seasons, comparable to an average increase of 44% in CH_4 emissions from rice paddies when the soil was warmed by 2 °C in a Japanese FACE study (Tokida et al., 2010, 2011; Liu et al., 2012). Several reasons may be given for the canopy warming-induced increase in CH_4 emissions. During the early rice-growing season under incomplete rice vegetative cover, canopy warming would increase soil temperature, and thereby even a moderate enhancement in organic matter decomposition due to soil warming can cause a large increase in CH_4 production given that Fe(III) reduction dominates electron-accepting processes (Tokida et al., 2010). During the late rice-growing season when soil temperature was not significantly increased (< 1 °C) with canopy warming due to complete rice vegetative cover (Gaihre et al., 2014; Liu et al., 2016a), however, the temperature effect on CH_4 emissions might have been more related to canopy warming on the photoassimilates allocation of rice (Cai et al., 2016). Previous results of

this FACE system showed that rising canopy temperature increased day respiration rate and leaf senescence of rice, shortened rice growth duration and decreased aboveground biomass and grain yield, suggesting that more photoassimilates could have been translocated to belowground and provided more C substrates available for methanogens (Table S1; Cai et al., 2016). In this study, rising canopy temperature shortened the preheading phase by 3.3 days and decreased grain yield by 23–39% (Cai et al., 2016). In addition, rising canopy temperature might have modified aerenchyma of rice plant such that it facilitated more rapid gaseous diffusion and CH_4 emissions from rice paddies. Nevertheless, an additive effect of elevated CO_2 and rising canopy temperature resulted in an increase of 82–143% in seasonal CH_4 emissions, slightly greater than an 80% increase due to the additive effects of elevated CO_2 and soil warming in a rice FACE study in Japan (Tokida et al., 2010).

4.2. Elevated CO_2 and rising temperature effects on methanogen abundance

An additive effect of elevated CO_2 and canopy warming on CH_4 emissions may be related to the response of methanogenic community to elevated CO_2 and canopy warming in rice paddies. Unfortunately, very few studies have linked soil methanogenic community to field CH_4 flux measurements in rice paddies (Liu et al., 2012), although some studies reported soil methanogens in rice paddies under elevated atmospheric CO_2 and increased soil temperature (Angel et al., 2012; Liu et al., 2016a, 2016b). Corresponding to seasonal dynamics of CH_4 fluxes, methanogen abundances of rhizosphere soil also showed a pronounced variation over the rice growing season in this study. Similar results were also found in previous studies (Das and Adhya, 2012; Ji et al., 2015), showing that artificial drying and rewetting resulted in a decrease of methanogen abundances in flooded rice fields.

Both elevated CO_2 and canopy warming significantly increased methanogen abundances of rhizosphere soil but their interaction was not pronounced in this study. The enhancement of methanogen abundance under elevated CO_2 was in agreement with most previous studies (Das and Adhya, 2012; Liu et al., 2016a; Li et al., 2017), which is primarily attributed to increased new C input into soil such as root exudates and rhizodeposition under elevated CO_2 (Conrad, 2007; Tokida et al., 2011). Cai et al. (2016) and Wang et al. (2016) reported that elevated CO_2 stimulated rice growth in this FACE experiment, which would increase rhizodeposition and root exudates in the rhizosphere (Bhattacharyya et al., 2013; Okubo et al., 2015). This is supported by the evidence that elevated CO_2 significantly increased rhizosphere soil organic C content over the 2014 rice-growing season in this study (Table S1).

In our study, rising canopy air temperature significantly increased the methanogen abundances in the rhizosphere soil of rice paddies (Fig. 3), in line with the results of soil warming studies (Das and Adhya, 2012; Liu et al., 2012). Liu et al. (2012) reported that rising soil temperature increased the root methanogen abundance in rice paddies. Das and Adhya (2012) showed that methanogenic population abundances increased with warming at intervals of ten degrees in trophic paddy soils. In general, rising temperature stimulates soil organic matter decomposition, which may provide more substrate available for methanogens under anaerobic conditions (Table S1). Besides, a significant interaction of temperature and stage on methanogen abundance revealed that rising canopy temperature-induced more photoassimilates could have been translocated to belowground and thereby increased soil organic C available for methanogens during the middle and late seasons (Table S1, Fig. 3).

4.3. Elevated CO_2 and rising temperature effects on methanogenic community composition

Methanogenic groups, including members of the orders *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*, *Methanopyrales* and *Methanocellales* have been identified from rice paddies (Ma et al., 2012; Liu et al., 2015; Lu et al., 2015; Alpana et al., 2017). In this study, methanogenic genera *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, *Methanocella* and *Methanoregula* belonged to the orders *Methanosarcinales*, *Methanobacteriales*, *Methanocellales*, and *Methanomicrobiales* were dominant in rhizosphere soil of rice paddies, while members of the orders *Methanococcales* and *Methanopyrales* were not identified (Table 4, Fig. 5). Similar results were also found in previous studies on methanogenic community of root and rhizosphere soil in rice paddies (Conrad et al., 2008; Liu et al., 2012; Breidenbach and Conrad, 2015; Lu et al., 2015; Liu et al., 2016a). Methanogens are typically separated into three categories, *i.e.*, hydrogenotrophic, acetoclastic and methylotrophic (Conrad, 2007; Liu and Whitman, 2008; Thauer et al., 2008; Lin et al., 2017). The co-existence of different categories of methanogen in rhizosphere soils suggest that acetate, H_2/CO_2 and methylated compounds were important carbon sources used for CH_4 production, while acetate could contribute most to CH_4 production since rhizosphere methanogenic communities were predominated by members of acetoclastic *Methanosaeta* (Watanabe et al., 2009; Liu et al., 2016a). Indeed, acetoclastic genera have been frequently found to be predominant methanogens in the soil of rice paddies in Asian countries (Conrad et al., 2012; Alpana et al., 2017).

In line with previous studies (Liu et al., 2012, 2016a, b), the overall distribution pattern and α-diversity of methanogenic community under genus level were not significantly affected by elevated CO_2, rising temperature and their interactions. However, elevated CO_2 altered the relative abundance of methanogenic genus, leading to a shift from acetoclastic to hydrogenotrophic methanogens in rhizosphere soils (Table 3, Fig. 5). It is generally believed that acetoclastic methanogens predominate in rice paddy while hydrogenotrophic methanogens can play a more predominant role in future climate scenario with elevated atmospheric CO_2 (Conrad, 2007; Das and Adhya, 2012). Rising temperature did not change the relative abundance of methanogen genus. Instead, it increased CH_4 production efficiency per predominate methanogen genus *Methanosaeta* in this study. This finding is consistent with the results of previous FACE studies, showing that soil warming led to a rise in activity of dominant methanogens in roots (Liu et al., 2012). Among soil physicochemical properties, soil organic matter (OM) contributed most to the difference in methanogenic community structure among the treatments (Fig. S1).

Table 4
OTUs and methanogens classified at the genus level that were retrieved from the rhizosphere soil of rice paddies in a FACE system.

OTU	Order	Family	Genus
OTU311	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*
OTU447	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*
OTU451	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*
OTU126	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*
OTU981	*Methanosarcinales*	Unclassified	
OTU54	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*
OTU94	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*
OTU111	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*
OTU634	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*
OTU49	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*
OTU92	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*
OTU521	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*
OTU728	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*
OTU299	*Methanocellales*	*Methanocellaceae*	*Methanocella* (Rice_Cluster_I)
OTU420	*Methanocellales*	*Methanocellaceae*	*Methanocella* (Rice_Cluster_I)
OTU560	*Methanocellales*	*Methanocellaceae*	*Methanocella* (Rice_Cluster_I)
OTU701	*Methanocellales*	*Methanocellaceae*	*Methanocella* (Rice_Cluster_I)
OTU186	*Methanomicrobiales*	*Methanoregulaceae*	*Methanoregula*
OTU672	*Methanomicrobiales*	*Methanoregulaceae*	*Methanoregula*
OTU913	*Methanomicrobiales*	Unclassified	

4.4. Limitation of this study

Relative to a previous study only examining methanogenic community but not linked to CH_4 fluxes in rice FACE system (Liu et al., 2016a), this study presented a linkage between the abundance and composition of methanogenic archaea and CH_4 fluxes as their responses to elevated CO_2 and rising canopy temperature in rice paddies. Obviously, there are some limitations in this FACE study. First, the abundance and composition of methanogenic community linking to CH_4 fluxes was examined only in the 2014 season, rather than over the 2012–2014 rice growing seasons. However, the response of CH_4 fluxes to elevated CO_2 and canopy warming did not significantly differ among the experimental years. Second, we only collected rhizosphere soil samples to investigate methanogenic community responses to elevated CO_2 and rising temperature. Some studies showed the difference in methanogenic community among bulk soils, rhizosphere soils and roots in rice paddies (Liu et al., 2012, Lee et al., 2015) and in freshwater marshes (Lin et al., 2017), while these available studies all demonstrated that methanogens were much more abundant in rhizosphere soils or roots than in bulk soils. In addition, we did not investigate methanotrophic community in this study, while our previous study found that the decreases in methanotrophs abundance were also related to increased CH_4 emissions from rice paddies under elevated CO_2 and rising temperature (Li et al., 2017). Nevertheless, these limitations of this study deserve to be further addressed in rice FACE studies.

5. Conclusion

Elevated CO_2 or rising temperature did not significantly alter seasonal pattern of CH_4 fluxes and methanogen abundances while elevated CO_2 and rising temperature had additive effects on seasonal CH_4 emissions and rhizosphere methanogen abundances. Either elevated CO_2 or rising temperature did not significantly alter the diversity of methanogenic community. However, elevated CO_2 induced a shift from acetoclastic to hydrogenotrophic methanogens in their relative abundances. Rising temperature stimulated CH_4 emissions by increasing CH_4 production per individual predominant methanogen genus. Overall, an additive effect of elevated CO_2 and rising temperature on CH_4 emissions from rice paddies was associated with the enhancement of soil C substrates and increase in methanogen abundances of rhizosphere soil, where elevated CO_2 changed the composition of methanogenic archaea while rising temperature stimulated the activity of methanogenic archaea. Given that both elevated CO_2 and rising temperature can alter soil GHGs fluxes, leading to a feedback to climate change, predicting this feedback should more concentrate on the microbial mechanisms involved in GHGs fluxes driven by climate change.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.agee.2018.02.003.

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